Form PTO 1390 (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER P32221
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/868394
INTERNATIONAL APPLICATION NO. PCT/GB99/04326	INTERNATIONAL FILING DATE 20 December 1999	PRIORITY DATE CLAIMED 18 December 1998
TITLE OF INVENTION NOVEL METHOD		
APPLICANT(S) FOR DO/EO/US Celia BRISCOE		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1 [x] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [x] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [x] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [x] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [x] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S. C. 371(c)(3)).

9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [x] An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98; and Form PTO-1449.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. [x] A FIRST preliminary amendment.

14. [ ] A SECOND or SUBSEQUENT preliminary amendment.

15. [x] Please amend the specification by inserting before the first line the sentence: This is a 371 of International Application PCT/GB99/04326, filed December 20, 1999, which claims benefit from the following Provisional Application: GB 9828087.8, filed December 18, 1998.

16. [ ] A substitute specification.

17. [ ] A change of power of attorney and/or address letter.

18. [x] An Abstract on a separate sheet of paper.

19. [x] Other items or information: Sequence Listing, Statement to Support, Diskette, Copy of Notification of Transmittal of International Search Report and International Search Report

US APPLICATION NO. (if known see 37 CFR 1.50)	INTERNATIONAL APPLICATION NO.	ATTORNEYS DOCKET NO.
09/868394	PCT/GB99/04326	P32221

20. [X] The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 C.F.R. 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO **$860.00**		
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) **$690.00**		
No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) **$710.00**	**$710.00**	
Neither International Preliminary Examination Fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....**$1,000.00**		
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$710.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**$0.00**	

Claims	Number Filed	Number Extra	Rate		
Total claims	**11 - 20 =**	**0**	**0 x $18.00**	**$0.00**	
Independent claims	**1 - 3 =**	**0**	**0 x $80.00**	**$0.00**	
Multiple dependent claims (if applicable)			**+ $270.00**	**$270.00**	
			TOTAL OF ABOVE CALCULATIONS =	**$270.00**	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				**$**	
			SUBTOTAL =	**$980.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				**$**	
			TOTAL NATIONAL FEE =	**$980.00**	
				Amount to be refunded	$
				charged	$

a. ☐ A check in the amount of $____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 19-2570 in the amount of **$980.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-2570 . A duplicate copy of this sheet is enclosed.

d. ☒ General Authorization to charge any and all fees under 37 CFR 1.16 or 1.17, including petitions for extension of time relating to this application (37 CFR 1.136 (a)(3)).

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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SIGNATURE
William T. Han
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34,344
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PATENT
ATTORNEY'S DOCKET NUMBER P32221

TRANSMITTAL LETTER TO THE U.S. DESIGNATED OFFICE
(DO/US) - ENTRY INTO NATIONAL STAGE UNDER 35 USC 371

INTERNATIONAL APP. NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB99/04326	20 December 1999	18 December 1998

TITLE OF INVENTION
NOVEL METHOD

APPLICANT(S) FOR DO/US
Celia BRISCOE

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231
ATTENTION: DO/US

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this Transmittal Letter, Form PTO 1390 and the papers indicated as being transmitted therewith, and Post Card are being deposited with the United States Postal Service on this date June 18, 2001 in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL737849063US addressed to the:

Assistant Commissioner for Patents, Washington, D.C. 20231.

Sabrina Smith
(Typed or printed name of person mailing paper)

Sabrina Smith
(Signature of person mailing paper)

"EXPRESS MAIL CERTIFICATE"
"EXPRESS MAIL" MAILING LABEL NUMBER EL737849063US
DATE OF DEPOSIT June 18, 2001

Attorney Docket No.: P32221

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Celia Briscoe	June 18, 2001	
Intl. App. No.:	PCT/GB99/04326	Group Art Unit:	Not Yet Assigned
Filed:	Herewith	Examiner:	Not Yet Assigned
For:	"Novel Methods"		

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 CFR §§ 1.821 THROUGH 1.825

BOX SEQUENCE
Assistant Commissioner for Patents
Washington, D.C. 20231

(X) I hereby state that the contents of the paper and computer readable copies of the Sequence Listing, submitted in accordance with **37 CFR §1.821(c)** and **(e)**, respectively, are the same.

( ) I hereby state that the submission filed in accordance with **37 CFR §1.821 (g)** does not include new matter.

( ) I hereby state that the submission filed in accordance with **37 CFR §1.821 (h)** does not include new matter or go beyond the disclosure in the international application as filed.

( ) I hereby state that the amendments, made in accordance with **37 CFR §1.825 (a)**, included in the substitute sheet(s) of the Sequence Listing are supported in the application, as filed, at pages _____. I hereby state that the substitute sheet(s) of the Sequence Listing does (do) not include new matter.

( ) I hereby state that the substitute copy of the computer readable form, submitted in accordance with **37 CFR §1.825(b)**, is the same as the amended Sequence Listing.

Serial No.: PCT/GB99/04326
Group Art Unit No.: Not Yet Assigned

(X) I hereby state that the substitute copy of the computer readable form, submitted in accordance with **37 CFR §1.825(d)**, is identical to that originally filed.

Respectfully submitted,

William T. Han
Attorney for Applicant
Registration No. 34,344

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SEQUENCE LISTING

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<120> Novel Method

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Attorney Docket No: P32221

IN THE UNITED STATES INTERNATIONAL EXAMINING AUTHORITY

Int'l. Appln. No.:	PCT/GB99/04326
Int'l. Filing Date:	20 December 1999
Priority Date:	18 December 1998
Applicant for DO/US:	Celia BRISCOE
Title of Invention:	NOVEL METHOD

Assistant Commissioner for Patents
Box PCT
Washington D.C. 20231

FIRST PRELIMINARY AMENDMENT

Sir:

Preliminary to calculating filing fees and examining this application, please amend the application as follows:

In the Specification:

Please replace the paragraph beginning at page 3, line 14, with the following rewritten paragraph:

-- Further suitable response elements include ATTTCCCCGAAAT (SEQ ID NO:1) (human and murine IRF-1, Pine, R., Canova, A. and Schindler, C. (1994) EMBO J. 13, 158-167.), ATTTCCCGTAAAT (SEQ ID NO:2) (human serum inducible element from the c-fos promoter, Zhong, Z., Wen, Z. and Darnell, J.E. Jr. (1994) Science 264, 95-98) ACTTCTTGGAATT (SEQ ID NO:3) (rat β-casein, Schindler, C., Kashleva, H., Pernis, A., Pine, R. and Rothman, P (1994) EMBO J. 13, 1350-1356) and ACTTCTAGGAATT (SEQ

ID NO:4) (bovine β-casein, Schindler, C., Kashleva, H., Pernis, A., Pine, R. and Rothman, P (1994) EMBO J. 13, 1350-1356). –

Please replace the paragraph beginning at page 7, line 22, with the following written paragraph:

-- The particular endothelial cells are transfected with a reporter plasmid, pGL2-basic luciferase vector (Promega) containing an insert of an oligonucleotide corresponding to a four fold tandem repeat of the STAT response element, ATTTCCCGTAAAT (SEQ ID NO:2), upstream of the minimal promoter for herpes simplex thymidine kinase (-35 to +10) using standard methodology for example the calcium phosphate method (Graham and Van Der Eb, Virology, 1973, **52**, 456). To correct for differences in transfection efficiency, the cells can be co-transfected with a reference plasmid expressing β-galactosidase activity. After a period of transfection (12-24 hours) the cells are treated with varying concentrations of compound or ob-protein alone as a positive control and then harvested and lysed. The lysates are assayed for luciferase, and if appropriate β-galactosidase, activity. Antagonist activity can be assayed by pre- or co-addition of an appropriate concentration of ob-protein to the compound under evaluation and measuring the reduction in luciferase response relative to that of ob-protein alone. Standard methods exist for assaying luciferase enzyme activity for example Ow et al., *Science*, 1986, **234**, 856 and de Wet et al., *Mol. Cell Biol.*, 1987, **7**, 725. as well as several commercial kits. --

In the Claims:

Please amend Claims 10 and 11 as follows:

10. (Amended) A method according to claim 9, wherein the response element is TTCCCGGAA (SEQ ID NO:5).

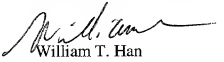
11. (Amended) A method according to claim 9, wherein the response element is selected from: ATTTCCCCGAAAT (SEQ ID NO:1), ATTTCCCGTAAAT (SEQ ID NO:2), ACTTCTTGGAATT (SEQ ID NO:3) and ACTTCTAGGAATT (SEQ ID NO:4).

REMARKS

This Preliminary Amendment is being made upon entry of International Application No. PCT/GB99/04326 into the U.S. national phase of prosecution in order to comply with the requirements of 37 CFR 1.821-1.825.

Attached hereto is a marked up version of the changes made to the specification and claims by the current amendment. The attached page is captioned **"Version with Markings to Show Changes".**

Respectfully submitted,



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VERSION WITH MARKINGS TO SHOW CHANGES

IN THE SPECIFICATION:

The paragraph beginning at page 3, line 14, has been amended as follows:

-- Further suitable response elements include ATTTCCCCGAAAT (SEQ ID NO:1) (human and murine IRF-1, Pine, R., Canova, A. and Schindler, C. (1994) EMBO J. 13, 158-167.), ATTTCCCGTAAAT (SEQ ID NO:2) (human serum inducible element from the c-fos promoter, Zhong, Z., Wen, Z. and Darnell, J.E. Jr. (1994) Science 264, 95-98) ACTTCTTGGAATT (SEQ ID NO:3) (rat β-casein, Schindler, C., Kashleva, H., Pernis, A., Pine, R. and Rothman, P (1994) EMBO J. 13, 1350-1356) and ACTTCTAGGAATT (SEQ ID NO:4) (bovine β-casein, Schindler, C., Kashleva, H., Pernis, A., Pine, R. and Rothman, P (1994) EMBO J. 13, 1350-1356). --

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-- The particular endothelial cells are transfected with a reporter plasmid, pGL2-basic luciferase vector (Promega) containing an insert of an oligonucleotide corresponding to a four fold tandem repeat of the STAT response element, ATTTCCCGTAAAT (SEQ ID NO:2), upstream of the minimal promoter for herpes simplex thymidine kinase (-35 to +10) using standard methodology for example the calcium phosphate method (Graham and Van Der Eb, Virology, 1973, **52**, 456). To correct for differences in transfection efficiency, the cells can be co-transfected with a reference plasmid expressing β-galactosidase activity. After a period of transfection (12-24 hours) the cells are treated with varying concentrations of compound or ob-protein alone as a positive control and then harvested and lysed. The lysates are assayed for luciferase, and if appropriate β-galactosidase, activity. Antagonist activity can be assayed by pre- or co-addition of an appropriate concentration of ob-protein to the compound under evaluation and measuring the reduction in luciferase response relative to that of ob-protein alone. Standard methods exist for assaying luciferase enzyme activity for example Ow et al., *Science*, 1986, **234**, 856 and de Wet et al., *Mol. Cell Biol.*, 1987, **7**, 725. as well as several commercial kits. --

IN THE CLAIMS:

Claims 10 and 11 have been amended as follows:

10. (Amended) A method according to claim 9, wherein the response element is TTCCCGGAA (SEQ ID NO:5).

11. (Amended) A method according to claim 9, wherein the response element is selected from: ATTTCCCCGAAAT (SEQ ID NO:1), ATTTCCCGTAAAT (SEQ ID NO:2), ACTTCTTGGAATT (SEQ ID NO:3) and ACTTCTAGGAATT (SEQ ID NO:4).

Novel Method

The invention relates to a novel method and more particularly to a method for the detection of compounds that mimic, potentiate or inhibit the physiological effects of the ob-protein

The ob-protein (or leptin) is a secreted hormone that acts as signal from adipose tissue to other organs to regulate weight and energy balance (Zhang et. al., *Nature*, 1994, **372**, 425). Additional roles for the ob-protein in haematopoietic and reproductive function have been suggested (Cioffi et. al. *Nature Medicine*, 1996, **2**(5), 585). Protein molecules that contain a core composed of four α-helices forming a bundle of up-up-down-down topology comprise a family of cytokines and growth factors. Proteins of this family cause homo- and hetero-oligomerisation of membrane receptors known to activate kinase cascades resulting in gene transcription. Receptors of the family which are activated by oligomerisation fall into two broad classes; those such as epidermal growth factor receptor, which possess integral tyrosine kinase activity in their intracellular domains (A. Ullrich & J. Schlessinger, *Cell*, 1990, **61**, 203-212), and those such as the IL4 and erythropoietin receptors, which lack this activity and mediate their response by way of an associated protein tyrosine kinase (J.N. Ihle et al., *TIBS*, 1994, **19**, 222-227). Both receptor subtypes are activated by cytokines, but the 4-helix bundle proteins activate only the non-integral tyrosine kinase subtype. The non-integral protein tyrosine kinase receptors generally act through a pathway involving Janus kinase (JAK) and their associated signal transducers and activators of transcription (STAT) proteins. On activation STAT proteins bind to DNA response elements thereby controlling gene transcription. Oligonucleotide sequences comprising DNA regulatory elements of the general sequence TT(N)nAA have been identified (Seidel et al., *Proc. Nat. Acad. Sci. USA.*, 1995, **92**, 3041) as STAT response elements. These elements bind STAT proteins in response to signalling molecules such as cytokines.

In copending United Kingdom patent application number 9509164.1 we have described our discovery that the ob-protein is characterised by a four helix bundle tertiary structure. We now believe that the ob-protein interacts with a membrane bound receptor that activates a JAK-STAT kinase cascade and hence forms the basis for an assay system for the detection of compounds that mimic, potentiate or inhibit the physiological effects of the ob-protein. Such an assay has utility in selecting compounds for the treatment of weight, energy balance, haematopoietic, fertility and other disorders modulated by the ob-protein. The assay is especially useful for selecting compounds for the treatment of those disorders related to obesity, anorexia, cachexia and diabetes.

Copending International patent application number PCT/EP96/02291 relates to a novel detection method which uses JAK-STAT technology. We have now found a particularly advantageous detection method which also utilises this technology.

Accordingly, the invention provides a method for the detection of a compound that mimics, potentiates or inhibits the physiological effect of the ob-protein, which method comprises:

(a) for a compound which mimics the physiological effect of the ob-protein, assessing the effect of the compound upon an ob-protein activated signal transducer and activator of transcription (STAT) DNA response element coupled to a reporter gene; or

(b) for a compound which potentiates or inhibits the physiological effect of the ob-protein, assessing the effect of the compound upon the response provided by ob protein upon an ob-protein activated STAT DNA response element coupled to a reporter gene;

wherein,

the response element and the reporter are expressed in an ob-protein responsive cell line or ob-protein responsive cells, which cell line is an endothelium derived cell line and which cells are endothelium derived cells.

A suitable source of endothelium-derived cells is a human immortalised endothelial cell line, a murine or other non-human immortalised endothelial cell line, human primary endothelial cells, or murine or other non-human primary endothelial cells.

A suitable human endothelium derived cell line is an ECV304-human umbilical cord cell line (see In Vitro Cell Dev. Biol. 1990; 26, 265; In Vitro Cell Dev Biol. 1991; 27A, 766 or In Vitro Cell Dev Biol. 1992; 28A, 380).

A suitable murine endothelial cell line is selected from the list consisting of:

SVEC4-10 –endothelial lymph node cells, SV40 transformed;
SVEC4-10EE2 –endothelial lymph node cells, SV40 transformed;
SVEC-10EHR1 - endothelial lymph node cells, SV40 transformed;
IP-1B - endothelial lymph node cells, SV40 transformed;
2F-2B - endothelial lymph node cells, SV40 transformed;
3B-11 - endothelial lymph node cells, SV40 transformed;
2H-11 -endothelial lymph node cells, SV40 transformed; and
MS1 (Mile SVEN 1)-endothelial pancreatic islet cells, SV40 transformed.

For SVE C4-10 see J. Immunol. 1990; 144, 521-525; Am. J. Pathol. 1991; 139, 743-749; J. Invest. Dermatol. 1993; 100, 742-745. SVEC4-10 is the parental cell line for a series of endothelial cell lines including; SVEC4-10EE2; SVEC-10HER; 2H-11; 3B-11; 2F-2B; and IP-1B. For MS1 see Proc. Natl. Acad. Sci. 1997, 94, 861-866.

Suitable human primary endothelial cells are selected from the list consisting of:

HUVEC - human umbilical vein endothelial cells;
HUAEC - human umbilical artery endothelial cells;
HAEC – human aortic endothelial cells;
HPAEC – human pulmonary artery endothelial cells;
HDMECa – human microvascular endothelial cells, adult dermis; and
HDMECn – human microvascular endothelial cells, neonatal dermis.

All of the above mentioned primary cells are commercially available.

A suitable polypeptide which is capable of mediating the stimulation by ob-protein of an ob-protein activated STAT DNA response element is a functional

isoform of the ob-gene receptor, for example that identified in Tartaglia et al., *Cell*, 1995, **83**, 1263.

Suitably, the response element is coupled to a promoter gene, preferably a minimal promoter.

A suitable response element is a nucleotide of formula $TT(N)_n$ AA, where N is any nucleotide and n is 4, 5 or 6.

A favoured response element is selectively activated by the intracellular events mediated the by the ob-protein interacting with its receptor. Such selective response elements can be determined by examining the relative activation of a range of response element-reporter gene constructs when transfected into an ob-responsive cell line by the ob-protein versus other cytokines.

A favoured response element is a nucleotide of formula $TT(N)_n$ AA, where N is any nucleotide and n is 4 or 5.

Further suitable response elements include ATTTCCCCGAAAT (human and murine IRF-1, Pine, R., Canova, A. and Schindler, C. (1994) EMBO J. 13, 158-167.), ATTTCCCGTAAAT (human serum inducible element from the c-fos promoter, Zhong, Z., Wen, Z. and Darnell, J.E. Jr. (1994) Science 264, 95-98) ACTTCTTGGAATT (rat β-casein, Schindler, C., Kashleva, H., Pernis, A., Pine, R. and Rothman, P (1994) EMBO J. 13, 1350-1356) and ACTTCTAGGAATT (bovine β-casein, Schindler, C., Kashleva, H., Pernis, A., Pine, R. and Rothman, P (1994) EMBO J. 13, 1350-1356).

Yet a further suitable response element is that region of the promoter of a gene regulated by the ob-protein that is required for STAT interactions. This gene will depend on the particular therapeutic use of the compounds to be selected by the assay.

A suitable reporter gene is firefly luciferase or chloramphenicol acetyltransferase enzyme.

A suitable promoter is a minimal promoter such as the herpes simplex virus thymidine kinase or SV40 promoter.

Other responsive cell lines can be identified using a displacement binding assay. Although binding may not be to a functional long form of the receptor, which is the form that transmits a signal to the cytoplasm. Identification of a functional long form of the receptor may be by PCR or Northern blot analysis (eg. Human ob-receptor: Tartaglia et al., *Cell*, 1995, **83**, 1263). Ultimately responsive cells are detected by monitoring cellular events in the presence of varying concentrations of leptin. Potential methods for identifying candidate cell lines or monitoring these cellular events include the following:-

1. Microphysiometer: This method detects small changes in pH resulting from biochemical changes in the cell. Ob-protein responsive cells upon stimulation may undergo biochemical changes that cause a small change in the extracellular acidification rate which can be detected by a silicon microphysiometer. The microphysiometer biosensor methodology has been reviewed by McConnell, *Science*, 1992, **257**, 1906.

2. Electrophoretic mobility shift assay (EMSA): Nuclear extracts from cells after treatment with ob-protein are mixed with radiolabeled oligonucleotides

containing a promiscuous or specific STAT response element DNA sequence. Extracts from cells that respond to the ob-protein may cause a gel shift of the oligonucleotide for the STAT response element.

References: Book "Recombinant DNA", 2nd Edition, Watson et al. , 1992, Page 158; Lamb et al., *Blood*, 1994, **83**, 2063;

3. Measurement of protein phosphorylation assay: The coupling of receptor activation to the final response through tyrosine phosphorylation of intracellular proteins may be assayed by the use of antibodies recognising phosphorylated tyrosines. More specifically since the leptin receptor may stimulate tyrosine phosphorylation of the JAK/STAT pathway this method provides a method of detecting leptin response cell lines. Specific JAK/ STAT antibodies may be used alongside antibodies for tyrosine phosphorylation to detect leptin activation in a leptin responsive cell line. Inhibition as well as stimulation of protein phosphorylation may occur. In particular, inhibition by the ob-protein of insulin stimulated phosphorylation of the insulin receptor and insulin receptor substrate-1 has been shown in rat-1 fibroblasts over expressing insulin receptors (Kroder et. al 1996, *Exp. Clin. Endocrinol. Diabetes*, **104**, suppl 2, p66)

4. Displacement binding: After incubation of cell lines with radiolabelled leptin, for example [^{125}I]-leptin, the non-specific binding versus specific binding of leptin can studied by the addition of unlabelled leptin. A high specific to non-specific ratio binding suggests that the cell line may contain the leptin receptor.

5. Detection of the protein for a functional form, preferably a functional long form, of the ob-receptor by use of selective antibodies.

6. Detection of mRNA for a functional form, preferably a functional long form, of the ob-receptor by Northern, RT-PCR or "slot blot" analysis.

7. Detection of increased c-fos mRNA after treatment with leptin. C-fos mRNA may be detected by Northern, RT-PCR or "slot blot" analysis.

Cell lines known to be involved in controlling aspects of the particular disease state for which compounds are being sought are preferred.

Cells lines derived from liver, brain, or pancreatic tissue and fibroblasts are particularly useful for "ob-responsive" cells for the assaying of compounds directed at obesity and diabetes. Certain areas of the brain are the focus of weight controlling and energy balance regulating effects of the ob-protein. The liver controls many metabolic processes that modulate lipid and glucose levels. Cells derived from particular regions of these organs containing the appropriate endogenous JAKs, STAT proteins and other intracellular proteins which are required for mediating the effects of the leptin are preferred.

The response element, the reporter, and preferably the promoter, are suitably incorporated into a vector capable of transfecting the ob-responsive cell line.

Suitable vectors are commercially available vectors, such as pGL2-basic luciferase vector (Promega).

A suitable configuration of the vector is the STAT DNA response element upstream of a promoter and a reporter gene. A more suitable configuration of the

vector is the STAT DNA response element in multiple tandem repeats (2-10) upstream of a thymidine kinase promoter and a luciferase reporter gene

Vectors are constructed containing a reporter gene for example firefly luciferase or chloramphenicol acetyltransferase enzyme linked to a minimal promoter for example the herpes simplex virus thymidine kinase or SV40 promoter. The DNA fragments for the STAT response element are inserted into the vector using appropriate restriction enzyme sites upstream of the minimal promoter.

The response element, the reporter and the promoter, as required, are incorporated into the vector using conventional expression techniques, for example the DNA fragments for the response element may be inserted into the vector using appropriate restriction enzyme sites upstream of the minimal promoter.

STAT response element-luciferase enzyme reporter systems can be constructed as described by Lamb et al., Blood, 1994, **8**, 2063 and Seidel et al., *Proc. Nat. Acad. Sci. USA.*, 1995, **92**, 3041.

Ob-responsive cells are transfected with the STAT response element-minimal promoter-luciferase reporter constructs using standard methodology for example the calcium phosphate method (Graham and Van Der Eb, *Virology*, 1973, **52**, 456). To correct for differences in transfection efficiency, the cells can be co-transfected with a reference plasmid expressing β-galactosidase activity. After a period of transfection (12-24 hours) the cells are treated with varying concentrations of compound and then harvested and lysed. The lysates are assayed for luciferase, and if appropriate β-galactosidase, activity. Potentiation or antagonist activity can be assayed by pre- or co-addition of an appropriate concentration of ob-protein to the compound under evaluation and measuring the potentiation or reduction in luciferase response relative to that of ob-protein alone. Standard methods exist for assaying luciferase enzyme activity for example Ow et al., *Science*, 1986, **234**, 856 and de Wet et al., *Mol. Cell Biol.*, 1987, **7**, 725. as well as several commercial kits.

Stable cell lines can be generated by transfecting an "ob-responsive" cell line with the reporter construct and a selectable marker. Selectable markers are routinely used to generate stable cell lines as described in Recombinant DNA, 2nd edition, J.D. Watson et. al., 1992, page 216. These stably transfected cell lines can be used to generate high throughput assays for compounds that mimic, potentiate or block the physiological effects of the ob-protein.

The invention also extends to a compound that mimics, potentiates or inhibits the physiological effect of the ob-protein, when identified by the method disclosed herein.

The invention also extends to a kit of parts adapted for use in the method disclosed herein.

When used herein 'a compound which mimics the physiological effects of the ob-protein' refers to a compound which is capable of acting in the absence of the ob-protein to either stimulate the ob-protein receptor to provide substantially the same physiological effect as the ob protein or to activate a response down stream of this receptor (post-receptor).

When used herein 'a compound that potentiates the physiological effect of the ob-protein' refers to a compound which enhances the potency and/or maximal physiological effect of the ob-protein.

When used herein 'a compound that inhibits the physiological effect of the ob-protein' refers to a compound which reduces or substantially blocks the physiological effect of the ob protein.

The cDNA encoding the functional form of the polypeptide can be transfected under the control of a constitutive promoter, (eg a viral promotor) or a regulatable promoter to optimise the expression of the polypeptide for the identification of agonists or antagonists as necessary. Alternatively, the response element and the reporter are expressed in a cell line, wherein a constitutive or regulatable promoter has been engineered into a position upstream of the chromosomally encoded gene for the ob-protein recepter by the method of homologous recombination. Such methods are reviewed by Waldman, Critical Reviews in Oncology/Hematology, 1992, 12, 49 and a particular example is given in te Riele et al, Proceedings of the National Academy of Sciences, 1992, 89, 5128.

The following examples illustrate the invention but do not limit it in any way.

Example

General Procedure:

Ob-responsive cells are transfected with a reporter plasmid containing a STAT response element, in multiple tandem copies upstream of a minimal promoter for example herpes simplex thymidine kinase and a luciferase gene reporter construct using standard methodology for example the calcium phosphate method (Graham and Van Der Eb, Virology, 1973, **52**, 456). To correct for differences in transfection efficiency, the cells can be co-transfected with a reference plasmid expressing β-galactosidase activity. After a period of transfection (12-24 hours) the cells are treated with varying concentrations of compound or ob-protein as a positive control and then harvested and lysed. The lysates are assayed for luciferase, and if appropriate β-galactosidase, activity. Antagonist activity can be assayed by pre- or co-addition of an appropriate concentration of ob-protein to the compound under evaluation and measuring the reduction in luciferase response relative to that of ob-protein alone. Standard methods exist for assaying luciferase enzyme activity for example Ow et al., *Science*, 1986, **234**, 856 and de Wet et al., 1987, **7**, 725. as well as several commercial kits.

Example 1

The particular endothelial cells are transfected with a reporter plasmid, pGL2-basic luciferase vector (Promega) containing an insert of an oligonucleotide corresponding to a four fold tandem repeat of the STAT response element, ATTTCCCGTAAAT, upstream of the minimal promoter for herpes simplex thymidine kinase (-35 to +10) using standard methodology for example the calcium phosphate method (Graham and Van Der Eb, Virology, 1973, **52**, 456). To correct for differences in transfection efficiency, the cells can be co-transfected with a reference plasmid expressing β-galactosidase activity. After a period of transfection (12-24 hours) the cells are treated with varying concentrations of compound or ob-protein alone as a positive control and then harvested and lysed. The lysates are assayed for luciferase, and if appropriate β-galactosidase, activity. Antagonist activity can be assayed by pre- or co-addition of an appropriate concentration of ob-protein to the compound under evaluation and measuring the reduction in luciferase response relative to that of ob-protein alone. Standard methods exist for assaying luciferase enzyme activity for example Ow et al., *Science*, 1986, **234**, 856 and de Wet et al., *Mol. Cell Biol.*, 1987, **7**, 725. as well as several commercial kits.

Claims

1. A method for the detection of a compound that mimics, potentiates or inhibits the physiological effect of the ob-protein, which method comprises:
(a) for a compound which mimics the physiological effect of the ob-protein, assessing the effect of the compound upon an ob-protein activated signal transducer and activator of transcription (STAT) DNA response element coupled to a reporter gene; or
(b) for a compound which potentiates or inhibits the physiological effect of the ob-protein, assessing the effect of the compound upon the response provided by ob protein upon an ob-protein activated STAT DNA response element coupled to a reporter gene;
wherein, the response element and the reporter are expressed in an ob-protein responsive cell line or ob-protein responsive cells, which cell line is an endothelium derived cell line and which cells are endothelium derived cells.

2. A method according to claim 1, wherein the endothelium-derived cell line is a human immortalised endothelial cell line, a murine or other non-human immortalised endothelial cell line.

3. A method according to claim 1 or claim 2, wherein the human endothelium derived cell line is an ECV304-human umbilical cord cell line.

4. A method according to any one of claims 1 to 3, wherein the murine endothelial cell line is selected from the list consisting of:
SVEC4-10 –endothelial lymph node cells, SV40 transformed;
SVEC4-10EE2 –endothelial lymph node cells, SV40 transformed;
SVEC-10EHR1 - endothelial lymph node cells, SV40 transformed;
IP-1B - endothelial lymph node cells, SV40 transformed;
2F-2B - endothelial lymph node cells, SV40 transformed;
3B-11 - endothelial lymph node cells, SV40 transformed;
2H-11 -endothelial lymph node cells, SV40 transformed; and
MS1 (Mile SVEN 1)-endothelial pancreatic islet cells, SV40 transformed.

5. A method according to claim 1, wherein the endothelium-derived cells are human primary endothelial cells, or murine or other non-human primary endothelial cells.

6. A method according to claim 1, wherein the endothelium-derived cells are selected from the list consisting of:
HUVEC - human umbilical vein endothelial cells;
HUAEC - human umbilical artery endothelial cells;
HAEC – human aortic endothelial cells;
HPAEC – human pulmonary artery endothelial cells;
HDMECa – human microvascular endothelial cells, adult dermis; and

HDMECn – human microvascular endothelial cells, neonatal dermis.

7. A method according to claim 1, wherein the polypeptide capable of stimulating an ob-protein activated STAT DNA response element is a functional isoform of the leptin receptor

8. A method according to claim 1, wherein the response element is coupled to a promoter gene, preferably a minimal promoter.

9. A method according to claim 1, wherein the response element is a nucleotide of formula $TT(N)_n AA$, where N is any nucleotide and n is 4, 5 or 6.

10. A method according to claim 9, wherein the response element is TTCCCGGAA.

11. A method according to claim 9, wherein the response element is selected from: ATTTCCCCGAAAT, ATTTCCCGTAAAT, ACTTCTTGGAATT and ACTTCTAGGAATT.

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

REPORTER GENE ASSAY FOR COMPOUNDS WHICH MIMIC OR INHIBIT THE PHYSIOLOGICAL EFFECT OF THE OB-PROTEIN

the specification of which (check one)

[ ] is attached hereto.

[X] was filed on 20 December 1999 as Serial No. PCT/GB99/04326
and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or Inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Filing Date	Priority Claimed
9828087.8	Great Britain	18 December 1998	Yes

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

Application Number	Filing Date

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Serial No.	Filing Date	Status

I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

Customer Number 20462.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

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